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Biochimica et Biophysica Acta 1758 (2006) 1696–1701
BIOCHIMICA ET BIOPHYSICA ACTA
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Histidine residues in the region between transmembrane domains III and IV of hZip1 are required for zinc transport across the plasma membrane in PC-3 cells

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Received 14 February 2006; received in revised form 11 May 2006; accepted 1 June 2006
Available online 7 June 2006

Abstract

The proteins from the ZIP and the CDF families of zinc transporters contain a histidine-rich sequence in a loop domain located between transmembrane domains III and IV for the ZIP family and transmembrane domains IV and V for the CDF family. Topological predictions suggest that these loops are located in the cytoplasm. The loops contain a histidine-rich sequence with a variable number of histidine residues depending on the transporter. The histidine-rich sequence was postulated to serve as an extra-membrane metal binding site in these proteins. hZip1 is a human zinc transporter ubiquitously expressed. The histidine-rich motif located in the large loop of this transporter is composed of the following sequence, $H_{158}WHD_{161}$. To determine if this motif is involved in the zinc transport activity of the protein, we performed site directed-mutagenesis to replace the loop histidines with alanines. Results suggest that both histidines are necessary for the zinc transport function and are not involved in the plasma membrane localization of the transporter as has been reported for the Zrt1 transporter in yeast. In addition, two histidine residues in transmembrane domains IV and V are also important in the zinc transport function. The results support an intermolecular exchange mechanism of zinc transport.

Keywords: Zinc transporter; hZip1; Zinc transport mechanism; SLC39A1

1. Introduction

The Zrt- and Irt-like Protein (ZIP) family and the Cation Diffusion Facilitator (CDF) family are composed of metal transporters involved in the uptake of metals into the cytosol and the efflux of metal from the cytoplasm, respectively. Proteins from both ZIP and CDF families contain a histidine-rich sequence located in a large loop domain between transmembrane domains (TM) III and IV for the ZIP family [1] and IV and V for the eukaryotic proteins from the CDF family [2]. It is interesting to note that despite the opposite function of the ZIP and CDF family transporters, topological predictions suggest that this large loop is located in the cytoplasm for both families. The histidine-rich sequence has a variable number of histidines and is postulated to serve as an extra-membrane metal binding site in these proteins [3]. Indeed, it has been shown for the histidine-rich motif of the rat transporter ZnT4 that it is capable of binding Zn^{2+}, Cu^{2+}, Ni^{2+} and Co^{2+} [4]. Metal uptake experiments in yeast by two variants of a nickel transporter (TgMTP1t1 and TgMTP1t2) from plants suggest that the histidine-rich motif is involved in metal specificity as the two variants differ only by the length of this motif [5]. Gitan et al. found that mutations of the 3 histidines in the loop of the yeast Zrt1 transporter cause a decrease in the Vmax but no change in the Km of zinc uptake. The mutations induce a mis-localization of Zrt1 in the yeast, leading to a decrease of the quantity of transporter present at the plasma membrane [6].

An earlier report indicated that transport of zinc by hZIP1 is not dependent on the free Zn^{2+} ion concentration, but depends on the total concentration of zinc present as Zn^{2+} and zinc

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doi:10.1016/j.bbamem.2006.06.005

bound to low molecular weight ligands; and that the mechanism of transport involves the intermolecular exchange of zinc between zinc ligands and the transporter [7]. Such an exchange mechanism likely involves a zinc-binding motif in the transporter. The histidine-rich motif located in the large loop of hZIP1 contains the sequence, $H_{158}WHD_{161}$. The coordination of the two histidines and the aspartate could create a zinc-binding site. Therefore, we wanted to determine if the histidine-rich motif in the loop of hZIP1 is involved in the transport activity and in the exchange of zinc with different zinc-ligands and whether the ligand form of zinc would affect the ability of the mutant hZIP1 transporter to uptake zinc.

2. Materials and methods

2.1. Plasmids and site-directed mutagenesis

The plasmid pcD-hZip1 consists of the hZip1 coding-sequence sub-cloned into pcDNA3 (Invitrogen) at the Not I restriction site. pcD-hZip1 was used as the template for site-directed mutagenesis. The mutants were generated using the site-directed mutagenesis kit QuikChange (Stratagene) following the manufacturer's protocol. The mismatch oligonucleotides used are shown in Table 1. After transformation, a few colonies for each mutant were confirmed by DNA sequencing. The plasmids containing the mutant hZip1 were named pcD-H158A (histidine 158 changed into alanine), pcD-H160A (histidine 160 changed into alanine), pcD-H158/160A (histidines 158 and 160 changed into alanines), pcD-H190A (histidine 190 changed into alanine) and pcD-H217A (histidine 217 changed into alanine). Subsequently, the different hZip1 inserts were transferred to the pCMV-MYC (Clontech) vector to create cMYC tag N-terminal fusion proteins of the transporters. The resulting constructs are pMYC-hZip1, pMYC-H158A, pMYC-H160A, pMYC-H158/160A, pMYC-H190A, and pMYC-H217A.

2.2. Cell culture and transfection

PC-3 cells (ATCC) were grown in RPMI medium supplemented with 10% fetal bovine serum (Gibco) and 1% Penicillin (10,000 units/ml)-Streptomycin (100,000 μg/ml). Cells were transfected using the Effectene kit from Qiagen and maintained in a humidified atmosphere of 5% CO_2. Stably transfected clones were selected by the dilution plating method in a medium containing 300 μg/ml of G418. PC-3 cells were also transiently transfected with the different MYC constructs using PolyFect reagent from Qiagen following the manufacturer's instructions.

2.3. Western blot

Proteins were extracted using a NP-40 buffer (50 mM Tris–HCl pH 7.5, 1% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF and a cocktail of antiproteases). Protein concentrations were determined using the BioRad protein assay based on the method of Bradford [8]. Equal amounts of protein were subjected to SDS-PAGE gel electrophoresis according to the method of Laemmli [9], transferred to nitrocellulose membranes and incubated overnight with anti-hZIP1 antibody [7] or anti-cMYC antibody (Sigma). The second antibody conjugated to HRP is either an anti-chicken IgY (Santa Cruz) for hZIP1 blots or anti-mouse IgG (Amersham Bioscience) for cMYC blots. The membranes were then stripped of antibodies and re-probed with anti-β-actin antibody (ICN).

Table 1
Primers used for site-directed mutagenesis of hZip1

H158A	GGTGGGCCGCAGGCTTGGCATGATGGGCCAGGG (forward)
	CCCTGGCCCATCAT GCCAAGCCTGCGGCCCACC (reverse)
H160A	GGTGGGCC GCAGCATTGGGCTGATGGGCCAGGG (forward)
	CCCTGGCCCATCAGCCCAATGCT GCGGCCCACC (reverse)
H158/160A	GGTGGGCCGCAGGCTTGGGCTGATGGGCCAGGG (forward)
	CCCTGGCCCATCAGCCCAAGCCTGCGG CCCACC (reverse)
H190A	CCCTGGCCCTCGCCTCCGTGTTCGAGGGG (forward)
	CCCCTCGAACACGGAGGCGAGGGCCAGGG (reverse)
H217A	GCTTTGCTGCTCGCCAAGGGCATCCTGGC (forward)
	GCCAGGATGCCCTTGGCGAGCAGCAAAGC (reverse)

2.4. ^{65}Zinc uptake assay

Cells were harvested by trypsin digestion from the flasks and washed once in PBS. They were then resuspended in cold uptake-buffer (Hanks Balanced Salt Solution, 50 mM HEPES, pH 7.4) and counted using a hemacytometer. Cell viability was determined by Trypan blue exclusion. In addition, cells from 50 μl samples were collected by centrifugation, proteins extracted and measured as described above. Uptake assays were initiated by the addition of 100 μl of cell suspension to 100 μl of uptake-buffer containing the specified concentration of $^{65}ZnCl_2$, ^{65}Zn-Citrate or ^{65}Zn-Cysteine with a ratio of metal/ligand equal to 1/3. The suspensions were incubated at 37 °C or 4 °C for the specified times. Assays were terminated by addition of four volumes of ice-cold stop-buffer (50 mM HEPES, 250 mM sucrose, 1 mM EDTA, pH 7.2). Cells were collected by filtration through glass fiber filters (Whatman GF/C) on a Brandel cell harvester. The filters were washed six times with wash-buffer (1× PBS with 1 mM EDTA) and the radioactivity counted in a liquid scintillation counter.

2.5. Immunohistochemistry

Thirty hours after transient transfection with the different cMYC tag constructs, PC-3 cells were treated for 1 h with 20 μM *N,N,N′,N′*-Tetrakis (2-pyridylmethyl) ethylene-diamine (TPEN). Cells were then fixed using 4% paraformaldehyde (PFA) for 20 min followed or not by permeabilization in 0.2% NP-40 for 5 min. Non-specific sites were blocked using 3% BSA. PC-3 cells were incubated with a cMYC antibody conjugated to fluorescein (Calbiochem) at a 1/100 dilution. The cover slips were mounted on slides using the ProLong® Gold antifade kit from Molecular Probe. The samples were analyzed with a confocal microscope, Zeiss 510 Meta.

3. Results

3.1. Expression of hZIP1 in PC-3 cells stably transfected

To determine if the histidine residues in the large loop between TM III and IV are involved in the zinc transport activity of hZIP1 (Fig. 1), we mutated the histidines in the loop to alanines, transfected PC-3 cells with the mutated constructs and selected cells that stably over expressed the mutated transporter proteins. Fig. 2 shows over expression of hZIP1 compared to non-transfected cells. The hZIP1 antibody detects a ~35 kDa protein in all of the cells, which is consistent with the molecular mass of hZIP1. This protein is over expressed in all of the transfected cells compared with non-transfected cells. The clone overexpressing wild type hZip1 has been previously reported [7].

3.2. Uptake of different forms of zinc

The transport of zinc across the plasma membrane by hZIP1 is not dependent on the free Zn^{2+} ion concentration, but is dependent on the total concentration of free and exchangeable zinc bound in complexes with various low molecular ligands [7]. The mechanism of transport appears to be an exchange between the zinc–ligand complexes and the hZIP1 transporter.

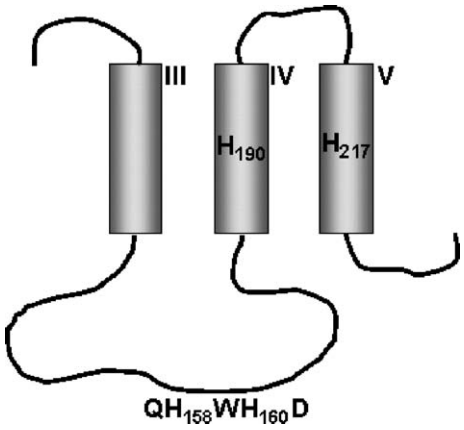


Fig. 1. Histidines mutated by site-directed mutagenesis in hZIP1. Transmembrane domains III, IV and V and the large loop of hZIP1 are represented. The histidines (H) shown are conserved in the transporters of the ZIP family. These residues were changed to alanines by site-directed mutagenesis.

The histidine motif in the loop is a potential zinc-binding site where exchange of zinc between the transporter and ligands could take place. To determine if this motif is involved in the mechanism of zinc transport, ^{65}Zn accumulation was measured in cells using 15 μM of three different forms of zinc: $ZnCl_2$ as free Zn^{2+} ion, Zn–Citrate (ZnCit) and Zn–Cysteine (ZnCys). ZnCit and ZnCys represent zinc complexes with log *K*f (formation constant) values of 5 and 10, respectively. At a Zn/Ligand ratio of 1/3, the concentrations of free Zn^{2+} ion for ZnCit and ZnCys are 3.8 μM and 0.8 pM, respectively (http://www.stanford.edu/~cpatton/webmaxc/webmaxcS.htm). The clone over-expressing wild type hZIP1 accumulated twice as much zinc as the non-transfected PC-3 cells with $ZnCl_2$, ZnCit or ZnCys as the zinc form. By contrast, PC-3 cells over-expressing the mutants H158A, H160A and H158/160A accumulated either less zinc or the same amount of zinc as the non-transfected PC-3 cells (Fig. 3). These results suggest that the histidine residues located in the cytoplasmic loop are involved in the transport function of hZIP1.

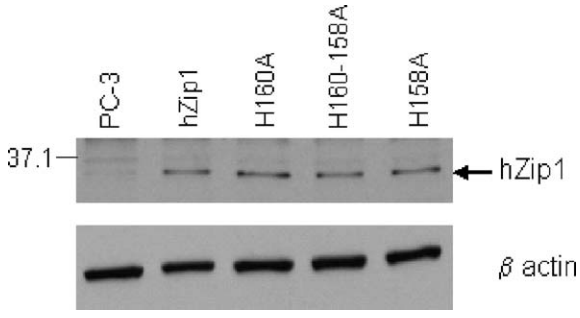


Fig. 2. Over-expression of hZIP1 in PC-3 cells. The cells used are non-transfected PC-3, stable transfectants over-expressing hZip1 and three different mutants H158A, H160A and H158/160A. Cells were harvested with trypsin and proteins from the whole cells were extracted with a NP-40 buffer. Western blot analyses were performed using an anti-hZIP1 antibody and an anti-β-actin antibody.

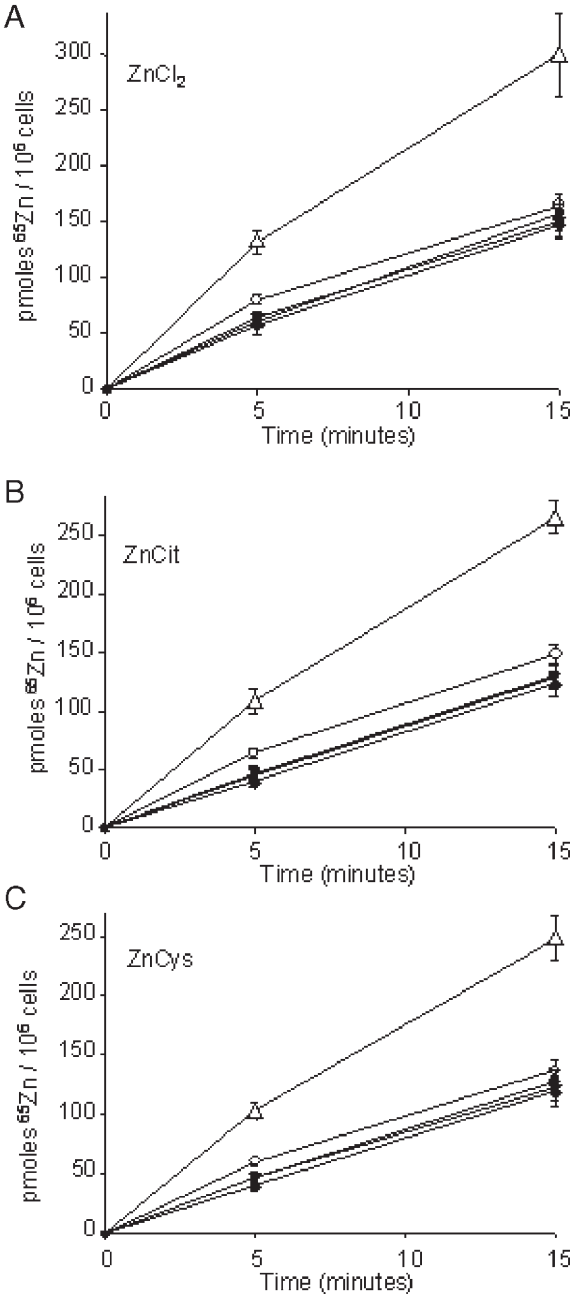


Fig. 3. Time course zinc uptake. ^{65}Zn uptake by non-transfected cells (open circles), hZip1 transfectant (open triangles), H158A mutant (closed squares), H160A transfectant (closed diamonds) and H158/160A transfectant (closed circles). Cells were incubated for 5 or 15 min with 15 μM of ^{65}Zn at 37 °C. A: $^{65}ZnCl_2$ uptake; B: $^{65}ZnCit$ uptake; C: $^{65}ZnCys$ uptake. Each value represents the mean±S.E. of a representative experiment (n=3).

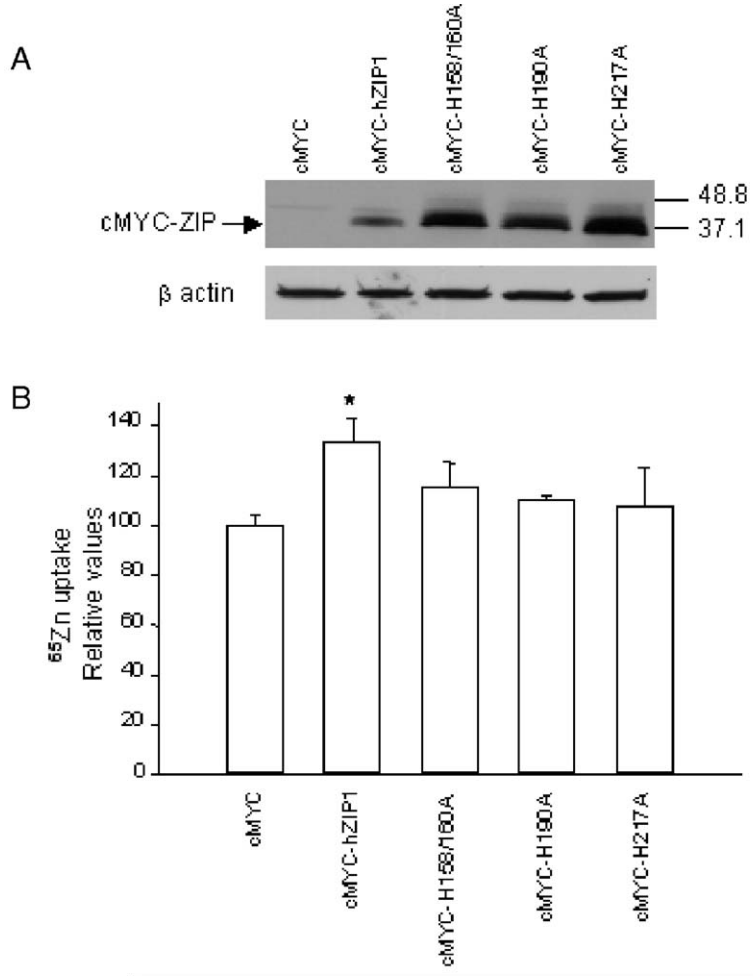
3.3. cMYC tagged hZIP1 fusion protein

The decrease in zinc uptake as a result of the histidine mutations could be caused by effects of the mutations on the localization of the mutated protein. Mutation of the histidine motif in the cytoplasmic loop of the Zrt1 zinc transporter in yeast resulted in reduced plasma membrane localization and accumulation of the protein in the cytoplasm [6]. To test this possibility we used a cMYC vector to express cMYC-hZIP1

fusion wild type and mutated proteins. The cMYC antigen epitope was placed at the N-terminal of the wild type and mutated forms of hZIP1. In addition, histidines 190 and 217 of hZIP1 located in transmembrane domains IV and V, respectively, were also mutated to alanine because the corresponding residues of the plant iron transporter Irt1 have been shown to be necessary for transport function [10]. ^{65}Zn uptake experiments were performed with PC-3 cells transiently transfected with constructs expressing the cMYC-hZIP1 fusion proteins (wild type or mutants). The expression of the fusion proteins was verified by western blot using an antibody directed against the cMYC tag (Fig. 4A). Although the mutant forms of the transporter were highly expressed, the expression of the wild type hZIP1 was always lower.

The results of the ^{65}Zn uptake experiments show that the wild type fusion protein is functional (Fig. 4B). Cells transfected with wild type hZIP1 fusion accumulated significantly more ^{65}Zn than cells transfected with the cMYC vector. On the other hand, although the fusion proteins with mutated hZIP1 are highly over-expressed compared to the wild-type fusion protein (Fig. 4B table), the uptake of zinc by these cells is the same as cells transfected with the cMYC vector. This suggests that histidines 158 and 160 in the loop and 190 and 217 in TM IV and V are important for the function of the transporter.

To determine the localization of the transporter, immunofluorescence staining was performed, using a fluorescein-conjugated antibody directed against the cMYC epitope. PC-3 cells transiently transfected with the different constructs were treated or not with TPEN for 1 h before fixation. TPEN has been shown to increase the amount of the transporter at the plasma membrane [11]. The results with permeabilized cells



	cMYC-hZIP1	cMYC-H158/160A	cMYC-H190A	cMYC-H217A
Level of expression	1.0 ± 0.19	6.1 ± 0.12	7.0 ± 1.8	9.0 ± 1.1

Fig. 4. Western blot analysis and $^{65}ZnCl_2$ accumulation with cMYC-hZIP1 fusion proteins. (A) A representative Western blot using a cMYC antibody to detect the expression of the fusion proteins is shown. (B) PC-3 cells transiently transfected with the different cMYC constructs were incubated for 15 min with 7 μM $^{65}ZnCl_2$. Each value represents the mean ± S.E. of two representative experiments ($n=6$), with the uptake for cMYC alone equal to 100. The asterisk represents a significant difference ($p<0.01$) with t-test in zinc uptake compared to cells transfected with cMYC alone. The table shows the relative level of expression of the different fusion proteins in the cells used for the zinc accumulation experiments. The values correspond to the band intensities corrected by β-actin band intensity from the same blot; cMYC-hZIP1 intensity is set equal to one.

show that the cMYC–hZIP1 fusion protein with the two histidines of the loop mutated is processed at the plasma membrane (Fig. 5, B and D). Plasma membrane staining is seen with or without TPEN treatment. Similar experiments with non-permeabilized cells also reveal staining at the plasma membrane for both mutated and non-mutated fusion proteins (data not shown).

4. Discussion

The maintenance of zinc homeostasis is critical to the growth and function of all cells. The intracellular zinc level is maintained through the activities of zinc importer and exporter plasma membrane proteins [12]. So far, most ZIP family members have been functionally identified as zinc import proteins [13]. Our previous studies established hZip1 (SLC39A1), a ubiquitous member of the family, as an important transporter for zinc uptake by prostate epithelial cells [7].

The conserved large cytoplasmic loop domain and the potential of the histidine residues in the loop to bind metal [4] suggest that this sequence and the loop might be important in the transport process. Previous work has shown that the transport mechanism involves the intermolecular exchange of zinc between zinc ligands and transporter [7]. The conserved histidine residues and the aspartate residue between TM III and IV is a potential region for such an exchange. In this report, we studied the role of the histidines in the TM III–IV loop region of hZIP1 in zinc uptake by the transporter. We utilized site-directed mutagenesis to change histidines 158 and 160 to alanines and determined the effects on zinc transport by PC-3 cells stably transfected with and over-expressing the mutant transporters.

The mutation of one or both histidines (158 and 160) to alanine resulted in a decrease in zinc accumulation by PC-3 cells transfected with the mutant transporters compared to PC-3 cells transfected with the wild type transporter. Consistent with our previous report, the ligand bound form of zinc had no effect on zinc accumulation. Since the concentration of free Zn^{2+} ion is 0.8 pM with ZnCysteine and 3.8 μM with ZnCitrate the zinc uptake was not dependent on the free Zn^{2+} ion concentration. These results suggest that these histidines are necessary for the normal function of the transporter. While it is clear that the mutant proteins are over expressed, the uptake of zinc is no different than for the non-transfected cells and less than that for cells over expressing the wild type protein. Moreover, the results show that each of the histidines is required for the transport function.

A previous report on the Zrt1 zinc transporter of yeast suggested that mutation of the histidine motif in the TM III–IV loop region interfered with the localization of the transporter to the plasma membrane and resulted in a 30% decrease of the Vmax compared to the wild type activity while the Km was not affected [6]. Our results showed that cells successfully transfected with the cMYC-hZIP1 mutated fusion protein displayed plasma membrane staining, indicating that the hZIP1 mutant is still processed at the plasma membrane of PC-3 cells.

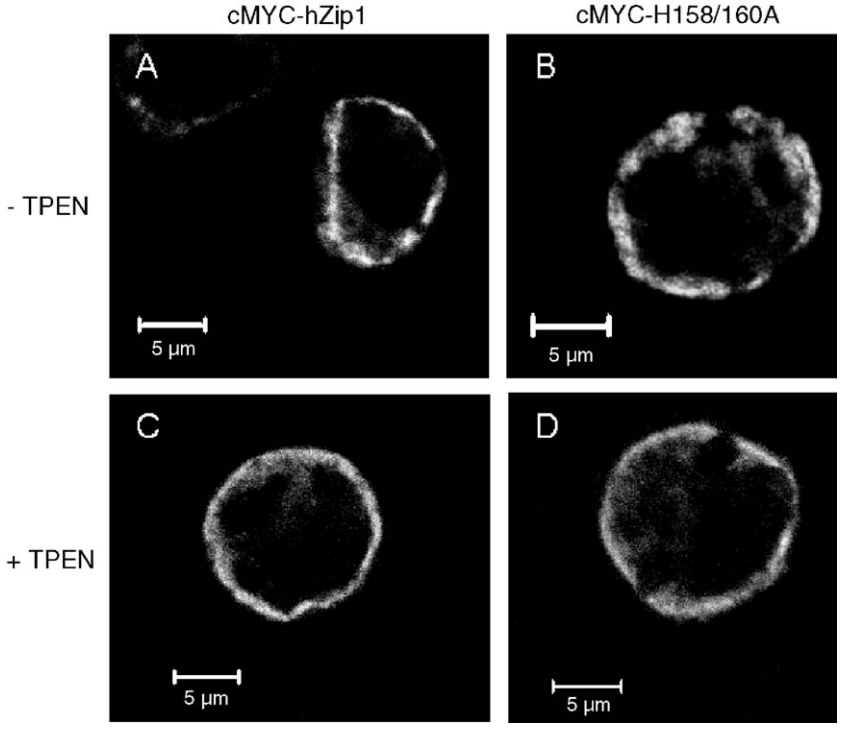


Fig. 5. Localization of the fusion protein cMYC-hZIP1 and cMYC-H158/160A in PC-3 cells. PC-3 cells transfected with cMYC-hZIP1 (A and C) and with cMYC-H158/160A (B and D) were treated (C and D) or not treated (A and B) with 20 μM TPEN before fixation and permeabilization. The fusion transporter is detected with a cMYC antibody conjugated with fluorescein. The staining was analyzed by confocal microscopy.

The zinc uptake experiments with cells stably transfected with the wild type hZip1 and the mutants show that the histidine-motif in the loop is involved in the zinc uptake activity of hZIP1. Apparently, the mutation of only one histidine is enough to impair the function. The experiments with the cMYC–hZIP1 fusion proteins confirm the importance of the motif located in the loop. Four types of zinc binding sites, structural, catalytic, cocatalytic and protein interface, have been described based on the three dimensional structure of known zinc binding proteins [14]. The two histidines and the aspartate comprising the histidine-rich motif of hZIP1 are spatially very close and zinc coordination under these conditions has not been described. Thus another possibility is that zinc binding by the histidine residues in the loop and the histidine residues in the transmembrane domains function to coordinate zinc during the transport process. As previously shown for Irt1, histidines 190 and 217 in TM IV and V of hZIP1, respectively, are important for the function of the transporter [10]. Thus the ternary structure of the transporter very likely brings the histidines of the loop into spatial proximity to the histidines in the transmembrane domains to bind zinc and allow transport across the membrane. The residues involved in zinc coordination and transport are located in a loop predicted to be intracellular; thus the issue of how extracellular ligands might exchange zinc with the transporter must be addressed. Reentrant loop topology has been described for several transporters [15–17]. We have evidence (unpublished data) that suggest that the large cytoplasmic loop of hZIP1 may be a reentrant loop. Our specific antibody is directed against a peptide located at the beginning of this loop. However, when cells are incubated with the antibody prior to zinc uptake measurements, a significant decrease in zinc accumulation by these cells is observed when compared to cells incubated with an antibody depleted preparation. This suggests that the epitope for the antibody is accessible from the outside of the cell and that the binding of the antibody to this epitope impairs hZIP1′s function. Thus the results of these studies are consistent with a transport mechanism that involves the intermolecular exchange of zinc between the transporter and zinc ligand complexes. Moreover, the results also suggest that the histidines in the loop between transmembrane domains III and IV are involved in zinc binding by the transporter. Such an intermolecular exchange mechanism of zinc transport could explain how zinc is transported under conditions where the concentration of free Zn^{2+} in extracellular fluid is extremely low.

Acknowledgment

This work was supported by the NIH Grant #NIH/NCI RO1 CA79903.

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